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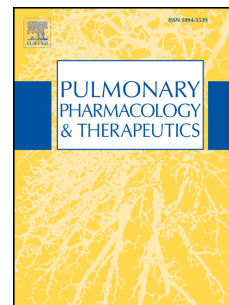
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Effect of lipopolysaccharide on the responsiveness of equine bronchial tissue

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Luigino Calzetta, Paola Rogliani and Elena Pistocchini contributed equally to the study.

Declaration of interest

None

Abbreviations

ACVIM: American College of Veterinary Internal Medicine; AHR: airways hyperresponsiveness; ANOVA: analysis of variance; ASICs: acid-sensitive ion

channels; ASM: airway smooth muscle; BALF: bronchoalveolar lavage fluid; CRCs: concentration-response curves; DMSO: dimethyl sulfoxide; EC_{50} : concentration inducing 50% E_{max} , EF_{50} : frequency inducing 50% E_{max} ; E_{max} : the maximal effect; electrical field stimulation (EFS); FRCs: frequency-response curves; IACUC: Institutional Animal Care and Use Committee; KH buffer solution: Krebs-Henseleit; LPS; lipopolysaccharide; MMPs: matrix metalloproteinases; NK_2 receptors: neurokinin-2 receptors; NKA: neurokinin A; RAO: recurrent airway obstruction; SEM: standard error of the mean; TRPV1: transient receptor potential vanilloid type 1 receptors.

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Abstract

Recurrent airway obstruction (RAO) is a main characteristic of horses with severe equine asthma syndrome. The presence of bacterial lipopolysaccharide (LPS) in the airways of horses is thought to play a crucial role in the clinical expression of this disorder. This study pharmacologically characterized the effect of LPS on the responsiveness of equine bronchial tissue. Equine isolated bronchi were incubated overnight with LPS (0.1-100 ng/ml) and then stimulated by electrical field stimulation (EFS). The role of capsaicin sensitive-sensory nerves (capsaicin desensitization treatment), neurokinin-2 (NK₂) receptors (blocked by GR159897), transient receptor potential vanilloid type 1 receptors (TRPV1; blocked by SB366791), and neurokinin A (NKA) were investigated. Untreated bronchi were used as control tissues. LPS (1 ng/ml) significantly increased the EFS-evoked contractility of equine bronchi compared with control tissues (+742±123 mg; $P<0.001$). At higher concentrations LPS induced desensitization to airways hyperresponsiveness (AHR; EC₅₀: 5.9±2.6 ng/ml). Capsaicin desensitization and GR159897 significantly prevented AHR induced by LPS at EFS_{1-50Hz} (-197±25%; $P<0.01$). SB366791 inhibited AHR at very low EFS frequency (EFS_{1Hz} -193±29%; $P<0.01$ vs. LPS-treated bronchi). LPS (1 ng/ml) significantly ($P<0.01$) increased 3.7±0.7 fold the release of NKA compared with control bronchi. LPS induces biphasic dysfunctional bronchial contractility due to the stimulation of capsaicin sensitive-sensory nerves, increased release of NKA, and activation of NK₂ receptors, whereas TRPV1 receptors appear to play a marginal role in this response. The overnight challenge with low concentrations of LPS represents a suitable model to investigate pharmacological options that may be of value in the treatment of equine RAO.

Keywords

Severe equine asthma syndrome, recurrent airway obstruction, airways hyperresponsiveness; airway smooth muscle; lipopolysaccharide; neurokinin A.

1. Introduction

Recurrent airway obstruction (RAO) is one of the main characteristics of horses with severe equine asthma syndrome, which has formerly been known as heaves [1]. RAO is characterized by cough, poor performance, increased mucus production, airway neutrophilic inflammation and airways hyperresponsiveness (AHR) leading to bronchial narrowing and airflow limitation. Horses affected by severe equine asthma syndrome show laboured breathing at rest following exposure to specific airborne agents, and reversible and reproducible airway obstruction related with the level of environmental exposures [1, 2]. Although in RAO-affected horses clinical remission can generally be achieved by moving horses to pasture or into an indoor low-airborne dust environment, prolonged and/or repeated exacerbations of severe equine asthma syndrome may lead to irreversible ultrastructural changes in the lung, and induce persistent abnormal contractility of airway smooth muscle (ASM), even when ill horses are maintained in a low dust environment [1, 3].

Although the specific antigens involved in triggering severe equine asthma syndrome have not yet been identified, exacerbation of clinical signs in RAO-affected horses are shown in animals stabled with straw bedding and fed hay, or following aerosolization of hay dust suspensions [4].

Indeed, it has been widely recognized that hay is the most common source of organic dust in stables containing both inhalable inorganic and organic particles such as moulds, mites, and bacterial lipopolysaccharide (LPS, also called bacterial endotoxin) [4], the latter playing a crucial role in the clinical expression of severe equine asthma syndrome [5-7]. Studies have demonstrated that the increase in the levels of matrix metalloproteinases (MMPs), proteolytic enzymes produced in the course of inflammation, found in bronchoalveolar lavage fluid (BALF) from severe asthmatic horses following inhalation challenge with LPS is dose-dependent [8], and that the inflammatory response in peripheral blood mononuclear cells from RAO-affected equines upon stimulation with hay dust extract might be a result of a synergistic interaction between LPS and certain allergens [4]. Intriguingly,

Pirie and colleagues reported that [9], while the acute administration of LPS increased the BALF neutrophil count in symptomatic horses in a dose dependent manner, only high doses of endotoxin induced a significant deterioration in lung mechanics. However, these authors correctly highlighted that the failure to detect airway bronchoconstriction could have been biased by the bronchodilatory effects of the α_2 -agonist drug used to sedate horses required in order to carry out the methacholine bronchoprovocation test [9].

Despite such a large body of evidence concerning the relevance of endotoxin in severe equine asthma syndrome, and the recent endorsement provided by the Consensus Statements of the American College of Veterinary Internal Medicine (ACVIM) [1], very little is currently understood about the effect of LPS on the responsiveness of bronchial tissue in horses and how this response can be pharmacologically manipulated. We have therefore hypothesized that LPS may induce neurogenic inflammation by stimulating capsaicin sensitive-sensory nerves in the lung [10] through the activation of the transient receptor potential vanilloid 1 (TRPV1) – neurokinin A (NKA) – neurokinin-2 (NK₂) receptor axis as has been suggested to occur in human airways [11].

2. Material and methods

2.1. Ethical approval

This study has been carried out in accordance with the EU Directive 2010/63/EU concerning the protection of animals used for scientific purposes. In order to not specifically sacrifice animals for our research aims, airway tissues have been obtained from horses killed in a slaughterhouse for food, in agreement with the current EU regulations 853/2004, 854/20014, 1069/2009, 142/2011, and 1069/2009. As such this study does not require to be consistent with the ARRIVE guidelines. However, this study has been approved by the local Ethics Committee (Institutional Animal Care and Use Committee [IACUC] of the University of Rome “Tor Vergata”, Rome, Italy).

2.2. Preparation of tissues

Equine airways were collected from 16 healthy horses (10 castrated male and 6 mares in anoestrus; aged 3.4 ± 0.3 years; weight 385.3 ± 29.5 kg). Tissues were placed into refrigerated ($4-6^{\circ}\text{C}$) Krebs-Henseleit (KH) buffer solution (NaCl, 119.0 mmol; KCl, 5.4 mmol; CaCl_2 , 2.5 mmol; KH_2PO_4 mmol, 1.2 mmol; MgSO_4 , 1.2 mmol; NaHCO_3 , 25.0 mmol; glucose, 11.7 mmol; pH 7.4) containing indomethacin ($5 \mu\text{M}$) and transported to the Laboratory of Respiratory Clinical Pharmacology at the University of Rome “Tor Vergata” (Italy) from a nearby abattoir. None of the animals were under pharmacological treatment or showed clinical or gross pathological signs of respiratory infections.

The experimental procedures began on the same day as the slaughter of the animals. In the laboratory, airways were cut into rings (sub-segmental bronchi; thickness 1-2 mm; internal diameter 4-6 mm) and transferred into a 10 ml High Tech 8 Channels Manual Compact Organ Bath system (Panlab Harvard Apparatus, Spain) containing KH buffer solution (37°C , pH 7.4) and aerated with O_2/CO_2 (95:5%) [12, 13]. Internal diameter and thickness of bronchial rings were measured via videomorphometry as previously described [14]. Tissues were allowed to equilibrate and the KH buffer solution was constantly changed.

2.3. Preparation of drugs

The following drugs were used: capsaicin (Sigma-Aldrich, Milan, Italy), GR159897 (Santa Cruz Biotechnology, Dallas, Texas, US), indomethacin (Sigma-Aldrich, Milan, Italy), LPS from *E. coli* 0111:B4 (Sigma-Aldrich, Milan, Italy), SB366791 (Santa Cruz Biotechnology, Texas, US). Capsaicin was dissolved in ethanol and then diluted in distilled water, indomethacin was dissolved in ethanol and then diluted in KH buffer solution, LPS was dissolved in distilled water, GR159897 and SB366791 were dissolved in dimethyl sulfoxide (DMSO). The maximal final concentrations of DMSO (0.1%) and ethanol (0.02%) achieved in the organ bath did not influence the response of isolated tissues, as previously reported [11, 15]. Appropriate dilutions were obtained in freshly prepared medium and stock solutions stored in small aliquots at -80°C until use.

2.4. Tension measurement

Bronchial rings were connected to isometric force transducers (Fort25, WPI, UK). The signal was amplified by a Powerlab 8/36 and Octal Bridge Amp system (AD instruments, UK), recorded and analyzed by using LabChart 7 interface software (AD instruments, UK). Tissues were mounted on hooks, and attached with thread to a stationary rod and the other end tied with thread to an isometric force displacement transducer. Airways were allowed to equilibrate for 90 min whilst flushing with fresh KH buffer solution every 10 min. Optimal passive tension was determined by gentle stretching of the tissues (resting tone: 2.0–2.5 g) during equilibration, as previously reported [15-18]. The isometric change in tension was measured by the transducer and the tissue responsiveness assessed by EFS delivered at 25 Hz. The rings were then washed three times and allowed to stabilize.

2.5. Transmural stimulation

EFS, also called transmural stimulation, was performed by placing tissues between two wire platinum electrodes (20 mm apart, Panlab Harvard Apparatus, Spain), connected to a 3165 multiplexing pulse booster stimulator (Ugo Basile, VA - Italy). In order to simulate ex vivo the vagal firing normally observed at the physiological frequency in vivo, bronchial rings were

contracted by EFS delivered at increasing frequencies (from 1 Hz to 50 Hz; 5V, 10s, 0.5ms, biphasic pulse), as described elsewhere [18-22]. The contractile responses of equine bronchi evoked by EFS are mediated through vagal nerve stimulation [23], as it is tetrodotoxin sensitive [24, 25].

2.6. Study design

Equine bronchial tissues were incubated overnight with KH buffer solution (control) or LPS administered at different concentrations (from 0.1 ng/ml to 100 ng/ml). Some LPS-incubated tissues were pre-treated with the potent and selective NK₂ receptor antagonist GR159897 administered at 0.3 μ M (pK_b: 8.57), or with the potent and selective transient receptor potential vanilloid type 1 (TRPV1) antagonist SB366791 administered at 2 μ M (pK_b: 7.74) [26, 27]. The final concentrations in the baths of antagonists used in this study were two logarithms greater than their pK_b and IC₅₀ values in order to selectively antagonize the targeted receptors [11].

In some experiments, the influence of capsaicin sensitive-sensory nerves was assessed with regard to the EFS-evoked bronchial contractility. Equine isolated airways were initially desensitized by five consecutive administrations of capsaicin (10 μ M, one hour apart from each other) as described elsewhere [15, 28], and then treated with LPS.

The day after these treatments, equine bronchial rings were mounted into the isolated organ bath system and connected to the isometric force transducers for recording the contractile response of airway smooth muscle (ASM) in response to EFS as previously described [20].

2.7. Neurokinin A quantification

The supernatant was collected in order to quantify the levels of neurokinin A (NKA) in response to LPS incubation. The release of NKA was measured by using a specific horse ELISA assay kit (MyBioSource, San Diego, CA, US) at 450 nm in 96-well plates, according to the manufacturers' instructions in triplicate experiments [11].

2.8. Data analysis

The contractile response of equine isolated airways was expressed in mg and the percentage of the contractile tone induced in control bronchi by each EFS frequency.

The frequency-response curves (FRCs) to EFS were analyzed by using appropriate sigmoid models to calculate the effect, the maximal effect (E_{\max}), and the frequency inducing 50% E_{\max} (EF_{50}). The fitting equation model was: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{Log}EF_{50} - X) * \text{HillSlope}))}$; where HillSlope described the steepness of the family of curves [29].

The concentration-response curves (CRCs) to LPS were also analyzed by using appropriate bell-shaped models to calculate the effect, the E_{\max} , and the concentration of LPS inducing 50% E_{\max} (EC_{50}). The fitting equation model was: $\text{Span1} = \text{Plateau1} - \text{Dip}$, $\text{Span2} = \text{Plateau2} - \text{Dip}$, $\text{Section1} = \text{Span1} / (1 + 10^{((\text{Log}EC_{50_1} - X) * nH1)})$, $\text{Section2} = \text{Span2} / (1 + 10^{((X - \text{Log}EC_{50_2}) * nH2)})$, $Y = \text{Dip} + \text{Section1} + \text{Section2}$; where Plateau1 and Plateau2 were the plateaus at the left and right ends of the curve, Dip was the plateau level in the middle of the curve, nH1 and nH2 were the HillSlopes [29].

The logarithm of the ratio between the ASM contractility in LPS-treated bronchi and that detected in control airways was also calculated in order to adequately quantify the stimulatory/inhibitory impact of the different concentrations of LPS on the contractile response of the bronchi to EFS [30].

Experiments were performed on $n=8$ bronchi collected from different horses and values are presented as mean \pm standard error of the mean (SEM). Each single treatment was carried out by using specimens collected from the same animal, and experiments were repeated 8 times in samples originating from 8 different horses. The levels of NKA were normalized for the wet weight of bronchial tissue and expressed as the ratio relative to the control bronchi.

Statistical significance was assessed by t-test and two-way analysis of variance (ANOVA) with Bonferroni post-tests carried out when necessary, and statistical significance defined as $P < 0.05$. Data analyses were performed using Prism 5 software (GraphPad Software Inc, La Jolla, CA, USA).

3. Results

3.1. Baseline characteristics

The baseline characteristics of the equine isolated airways used in this study are reported in Table 1. No significant baseline differences ($P>0.05$) were detected among the bronchial rings used in this study.

Table 1. Baseline characteristics of the bronchial rings used in the study and the contractile response induced by five consecutive capsaicin administrations ($10 \mu\text{M}$) at hourly intervals as the protocol to induce desensitization.

Wet weight (mg)	Internal diameter (mm)	Thickness (mm)	Resting tone (g)	Tissue responsiveness at EFS _{25Hz} (mg)
95.6±3.4	4.45±0.04	1.28±0.02	2.24±0.03	302.2±25.4
Capsaicin sensitive-sensory nerves desensitization (contractile response expressed as mg)				
1 st administration	2 nd administration	3 rd administration	4 th administration	5 th administration
1,990.5±399.4 ^a	1,120.7±228.8 ^b	610.2±121.0 ^c	360.5±82.0 ^c	60.4±13.3 ^c

a. $P<0.001$ vs. resting tone

b. $P<0.05$ vs. contractile response at 1st administration

c. $P<0.001$ vs. contractile response at 1st administration

Capsaicin ($10 \mu\text{M}$) induced a significant contractile response of equine isolated bronchi ($P<0.001$ vs. resting tone), and five consecutive capsaicin administrations resulted in a significant tachyphylaxis ($P<0.05$ vs. the first capsaicin administration) such that the last (5th) administration of capsaicin did not significantly modify the baseline bronchial tone ($P>0.05$ vs. resting tone) (Table 1).

The desensitization procedure of capsaicin sensitive-sensory nerves, GR159897 administered at $0.3 \mu\text{M}$ and SB366791 administered at $2 \mu\text{M}$ did not significantly modify ($P>0.05$) either the baseline bronchial tone or the contractile response to transmural stimulation in control bronchi (data not shown).

3.2. Impact of LPS on the FRC to transmural stimulation

The overnight incubation with LPS administered at 1 ng/ml elicited a significant ($P<0.001$) increase in bronchial contractility (E_{max} : $1,056.00\pm123.80 \text{ mg}$) induced by EFS_{1-50Hz} compared with control tissues (E_{max} : $314.50\pm16.45 \text{ mg}$). The overnight treatment of bronchial tissues with LPS administered at

0.1 ng/ml, 10 ng/ml and 100 ng/ml did not modify E_{\max} elicited by transmural stimulation. Overall, no concentration of LPS altered the EF_{50} compared with control tissues, that ranged between 6.75 Hz to 9.47 Hz (Figure 1).

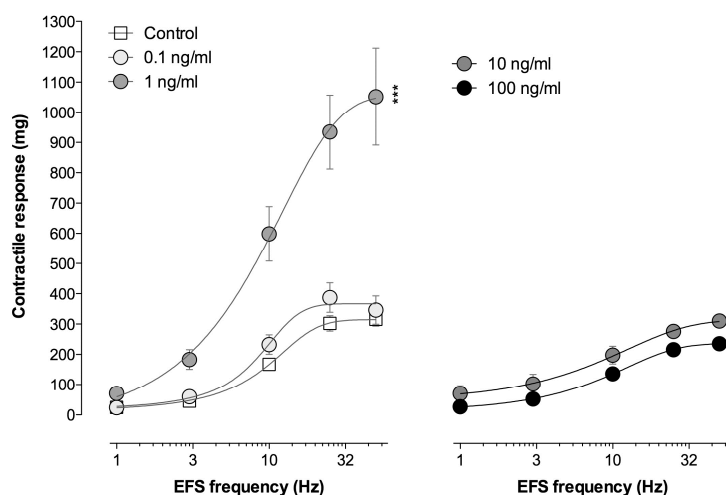


Figure 1. Impact of overnight incubation of equine isolated bronchi with different concentrations of LPS on the FRC to EFS. Points represent the mean \pm SEM of experiments performed in n=8 different isolated bronchi from 8 different horses. ***P<0.001 vs. control (statistical significance assessed by two-way ANOVA). EFS: electrical field stimulation; FRC: frequency response curve; LPS: lipopolysaccharide.

3.3. Impact of increasing concentrations LPS on the contractile response to transmural stimulation

The challenge of equine isolated bronchi with increasing concentrations of LPS elicited a bell-shaped contractile response to EFS. LPS induced a potent AHR when administered at low concentrations (overall EC_{50_1} : 0.46 ± 0.06 ng/ml; overall E_{\max} at 1 ng/ml: $+238.71\pm53.65\%$ vs. control; $P<0.05$), whereas at higher concentrations LPS elicited a progressive desensitization to AHR (overall EC_{50_2} : 5.85 ± 2.62 ng/ml) (Figure 2).

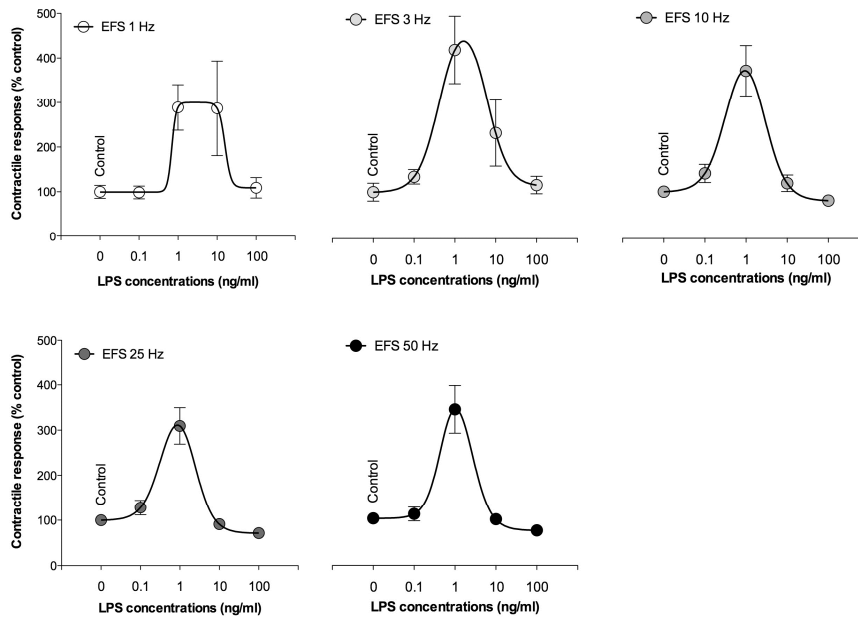


Figure 2. Impact of overnight incubation of equine isolated bronchi with increasing concentrations LPS on the contractile response to transmurial stimulation. Points represent the mean \pm SEM of experiments performed in n=8 different isolated bronchi from 8 different horses. EFS: electrical field stimulation; LPS: lipopolysaccharide.

Specifically, the logarithmic analysis of the ratio between the ASM contractility in LPS-treated bronchi and that detected in control airways showed that significant ($P<0.05$) AHR was induced by LPS administered at 1 ng/ml and 10 ng/ml when isolated bronchi were stimulated by low EFS frequencies (EFS_{1-3Hz}), and that significant ($P<0.001$) AHR to medium-high EFS frequencies (EFS_{10-50Hz}) was elicited only when LPS was administered at 1 ng/ml. Conversely, the overnight incubation of equine isolated airways with higher concentrations of LPS (100 ng/ml) induced a significant ($P<0.05$) desensitization of the ASM contractility elicited by medium-high EFS frequencies (EFS_{10-50Hz}) (Figure 3).

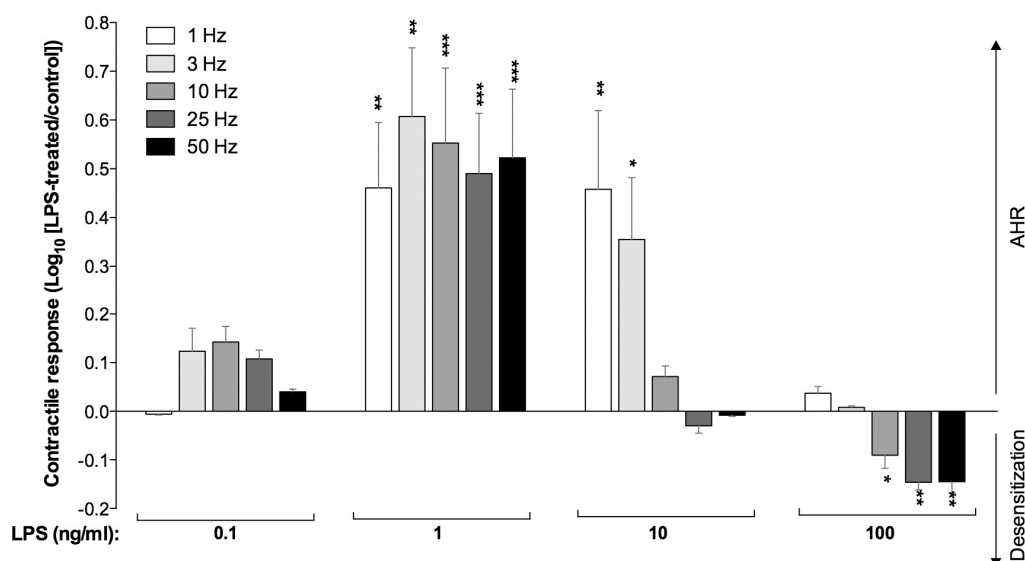


Figure 3. Fold changes expressed as logarithmic scale of the ratio between the ASM contractility induced by transmural stimulation in LPS-treated bronchi and that detected in control airways. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. unchanged ASM contractility (statistical significance assessed by two-way ANOVA with Bonferroni post-test). (Log_{10} : 0). Values > 0 indicate AHR, whereas values < 0 indicate desensitization to transmural stimulation. ASM: airway smooth muscle; AHR: airways hyperresponsiveness; LPS: lipopolysaccharide.

3.4. Mechanisms leading to AHR in LPS-incubated equine airways

The AHR induced in response to $\text{EFS}_{1-50\text{Hz}}$ induced by the overnight incubation of equine isolated airways with LPS administered at 1 ng/ml was significantly prevented by the desensitization of capsaicin sensitive-sensory nerves, as well as by specifically antagonising NK_2 receptors with GR159897 ($-180.14 \pm 22.43\%$ and -213.83 ± 28.02 , respectively; both $P < 0.01$ vs. LPS-treated bronchi). The selective antagonism of TRPV1 receptors with SB366791 significantly prevented the AHR induced in response to very low EFS frequencies ($\text{EFS}_{1\text{Hz}}$: $-193.15 \pm 29.38\%$; $P < 0.01$ vs. LPS-treated bronchi), but did not inhibit the AHR induced in response to higher EFS frequencies ($\text{EFS}_{3-50\text{Hz}}$: $+29.98 \pm 19.35\%$; $P > 0.05$ vs. LPS-treated bronchi) (Figure 4).

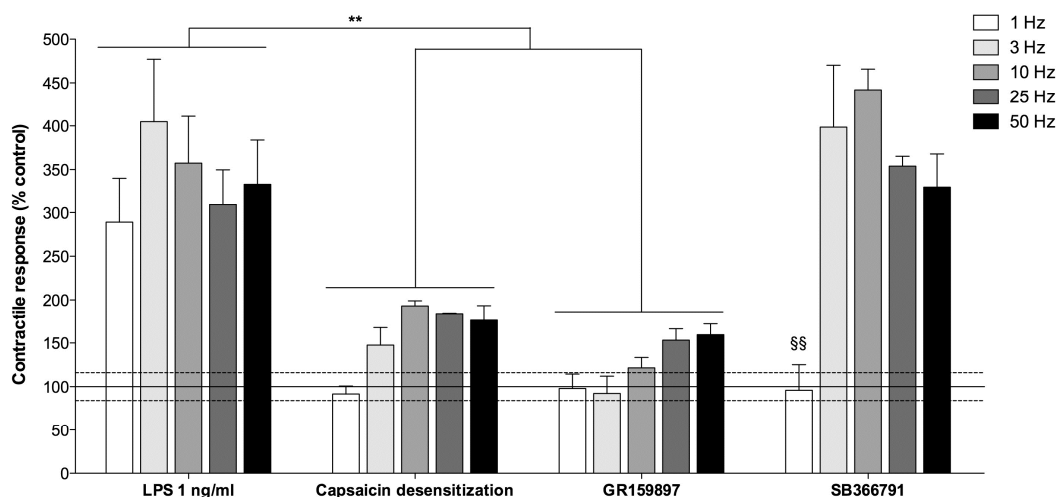


Figure 4. Influence of capsaicin sensitive-sensory nerves (capsaicin desensitization treatment), NK₂ receptor (blocked by GR159897), and TRPV1 (blocked by SB366791) on the AHR induced by LPS administered at 1 ng/ml on the transmural stimulation. The continuous line indicates the normalized contractility in control bronchi and SEM (dotted lines). **P<0.01 vs. LPS-treated bronchi (statistical significance assessed by two-way ANOVA) and §§P<0.01 vs. AHR induced by LPS on EFS_{1Hz} (statistical significance assessed by t-test). AHR: airways hyperresponsiveness; EFS: electrical field stimulation; LPS: lipopolysaccharide; NK: neurokinin; TRPV1: transient receptor potential vanilloid type 1.

The overnight incubation of equine bronchial rings with LPS administered at 1 ng/ml LPS significantly (P<0.01) increased 3.72±0.73 fold the release of NKA compared with control bronchi.

4. Discussion

The results of this study demonstrate for the first time that LPS may elicit both AHR and bronchial desensitization in equine airways, depending on the concentration. Specifically, overnight incubation with low concentrations (1 ng/ml) of LPS induced strong AHR in response to transmural stimulation. Conversely, the overnight challenge with LPS at higher concentrations (100 ng/ml) inhibited the bronchial contractility in response to medium-high EFS frequencies. The bronchial desensitization of equine airways induced by high concentrations of LPS confirms the results of our recent investigations carried out using human airways where we have demonstrated that the overnight treatment of human isolated bronchi with LPS administered at 100 ng/ml significantly inhibited the contractile tone in response to EFS_{25-50Hz} [20].

In the current study we found that the best fitting equation model to describe the impact of increasing concentrations of LPS on the EFS-evoked contractions was a bell-shaped CRC, in which the stimulatory response at low-medium concentrations was greater than that elicited at higher concentrations of endotoxin. Indeed, this pharmacological model is more complicated than the standard monotonic sigmoid CRCs, although in this study we have analyzed a sufficient number of data points to adequately define both phases of bronchial responsiveness.

The classic mechanisms through which bell-shaped CRCs may be understood include the administration of drugs that both activate and inhibit transmembrane receptors, and the complicated stimulus-response relationships caused by the promiscuity of different receptors [31, 32]. Our findings suggest that the bell-shaped CRCs induced by LPS is related with initial AHR following exposure to low concentrations, followed by a second desensitization phase following exposure to higher concentrations. This bell-shaped relationship seems to be due to the role of capsaicin sensitive-sensory nerves in the bronchial responsiveness to LPS as the desensitization of capsaicin sensitive-sensory nerves induced by 5 consecutive administrations of capsaicin abolished the AHR induced by LPS. This evidence suggests that LPS acts on capsaicin sensitive-sensory nerves, and

that while low concentrations of endotoxin elicit sensitization of equine airways, higher concentrations of LPS induce tachyphylaxis and thus bronchial desensitization to transmural stimulation. This evidence further supports the pivotal role of capsaicin sensitive-sensory nerves in the modulation of bronchial contractility as previously reported in both human and equine isolated airways in response to different stimuli [11, 15, 28, 33].

TRPV1 receptors have been suggested to play a crucial role in LPS-induced neurogenic inflammation, as LPS may directly and indirectly activate TRPV1 receptors, at least in human, bovine and murine tissues [11, 34-38]. Our results clearly indicate that activation of TRPV1 receptors has only a partial role in the AHR induced by LPS in equine airways. Indeed, although the selective TRPV1 antagonist SB366791 prevented the LPS-induced AHR to a very low EFS frequency (EFS_{1Hz}), it was ineffective in preventing the AHR to higher EFS frequencies (EFS_{3-50Hz}).

Our evidence suggests that the impact of LPS on the bronchial responsiveness to transmural stimulation is mainly TRPV1-independent. To date, little is known concerning the mechanisms for the TRPV1-independent activation of capsaicin sensitive-sensory nerves that lead to neurogenic inflammation [39], although the activation of acid-sensitive ion channels (ASICs) or certain types of voltage-gated potassium channel, the increase in oxidative stress, the inhibition of NADH-oxido-reductase system and of other enzymes [40-43] have all been suggested as possible mechanisms.

Certainly, the overnight challenge with LPS induces oxidative stress in human isolated airways by inhibiting the activity/levels of endogenous anti-oxidant agents, and by increasing the activity/levels of pro-oxidant factors [20]. Therefore, we cannot exclude that the same mechanisms may modulate the neurogenic inflammation observed in equine isolated bronchi. We have also demonstrated that the overnight challenge with LPS increases the release of NKA, and that blocking its specific receptor, NK_2 , by using the selective antagonist GR159897, prevents the AHR induced by endotoxin. Sensory neuropeptides, including NKA, are thought to be responsible for neurogenic inflammation in the respiratory tract [42, 44, 45], a condition that in equine

isolated airways facilitates the cholinergic pathways in the vagus nerve stimulated by a wide range of EFS frequencies. Therefore, we can conclude that the impact of LPS on the responsiveness of equine bronchi is mainly mediated by an increased activation of NK₂ receptors due to enhanced release of NKA, whereas the role of TRPV1 receptors is mainly relegated to the activation of capsaicin sensitive-sensory nerves stimulated by very low EFS frequencies.

This ex vivo study extends the study of Venugopal and colleagues concerning the modulation of neurokinin receptors in RAO-affected horses [46] who demonstrated that the expression of NK₂ receptor was up-regulated in bronchi of RAO-affected horses, and that isolated airways of RAO-affected subjects were more responsive to NKA compared with those of normal horses. These findings, together with the evidence provided by our current research, indicate that the environmental exposure to bacterial LPS may represent a crucial factor in modulating the bronchial responsiveness of severe asthmatic equines.

The biphasic effect of LPS on bronchial responsiveness of equine airways suggests that a sporadic and acute exposure to low concentrations of endotoxin in the lung may exert a protective bronchoconstriction at the level of sub-segmental bronchi that, in turn, prevents the delivery of LPS to small airways. Conversely, the chronic exposure to high concentrations of inhaled endotoxin may induce airway desensitization leading to dysfunctional response of ASM, a condition that allows LPS and further organic and inorganic particles to reach the distal airways and induce lung inflammation [1].

5. Conclusions

LPS has deleterious effects on equine airways by inducing dysfunctional ASM contractility due to the stimulation of capsaicin sensitive-sensory nerves, increased release of NKA, and activation of NK₂ receptors. The findings of this study also suggest that the impact of LPS on equine airways is only minimally dependent on the activation of TRPV1 receptors. Therefore, antagonising NK₂ receptors may have a beneficial impact at normalizing the ASM contractility and controlling clinical signs in severe asthmatic horses.

Although targeting neurokinin receptors failed to show any clinical benefit in humans with asthma [47-50], this study suggests that well designed clinical trials are needed to assess whether selective NK₂ receptor antagonists (i.e. SR48968) [51] and non-selective NK receptor antagonists (i.e. FK224, AVE5883, CS003, DNK333) [52-55] may have clinical effectiveness in equines with severe asthma.

Finally, but no less important, our results suggest that the overnight challenge with low concentrations of LPS as described in this study represents a suitable ex vivo model to investigate the impact of drugs as possible treatment approaches for equine RAO.

6. References

- [1] Couetil LL, Cardwell JM, Gerber V, Lavoie JP, Leguillette R, Richard EA. Inflammatory Airway Disease of Horses--Revised Consensus Statement. *J Vet Intern Med.* 2016;30:503-15.
- [2] Pirie RS, Couetil LL, Robinson NE, Lavoie JP. Equine asthma: An appropriate, translational and comprehensible terminology? *Equine Vet J.* 2016;48:403-5.
- [3] Pirie RS. Recurrent airway obstruction: a review. *Equine Vet J.* 2014;46:276-88.
- [4] Lanz S, Gerber V, Marti E, Rettmer H, Klukowska-Rotzler J, Gottstein B, et al. Effect of hay dust extract and cyathostomin antigen stimulation on cytokine expression by PBMC in horses with recurrent airway obstruction. *Vet Immunol Immunopathol.* 2013;155:229-37.
- [5] Pirie RS, Dixon PM, McGorum BC. Endotoxin contamination contributes to the pulmonary inflammatory and functional response to *Aspergillus fumigatus* extract inhalation in heaves horses. *Clin Exp Allergy.* 2003;33:1289-96.
- [6] Pirie RS, Collie DD, Dixon PM, McGorum BC. Inhaled endotoxin and organic dust particulates have synergistic proinflammatory effects in equine heaves (organic dust-induced asthma). *Clin Exp Allergy.* 2003;33:676-83.
- [7] Berndt A, Derksen FJ, Venta PJ, Ewart S, Yuzbasiyan-Gurkan V, Robinson NE. Elevated amount of Toll-like receptor 4 mRNA in bronchial epithelial cells is associated with airway inflammation in horses with recurrent airway obstruction. *Am J Physiol Lung Cell Mol Physiol.* 2007;292:L936-43.
- [8] Simonen-Jokinen T, Pirie RS, McGorum B, Maisi P. Dose responses to inhalation of endotoxin, hay dust suspension and *Aspergillus fumigatus* extract in horses as measured by levels and activation of matrix metalloproteinase-9. *Equine Vet J.* 2005;37:155-60.
- [9] Pirie RS, Dixon PM, Collie DD, McGorum BC. Pulmonary and systemic effects of inhaled endotoxin in control and heaves horses. *Equine Vet J.* 2001;33:311-8.
- [10] Groneberg DA, Quarcoo D, Frossard N, Fischer A. Neurogenic mechanisms in bronchial inflammatory diseases. *Allergy.* 2004;59:1139-52.
- [11] Calzetta L, Luongo L, Cazzola M, Page C, Rogliani P, Facciolo F, et al. Contribution of sensory nerves to LPS-induced hyperresponsiveness of human isolated bronchi. *Life Sci.* 2015;131:44-50.
- [12] Menozzi A, Pozzoli C, Poli E, Colla C, Placenza G, Bertini S. Effects of selective α_2 -adrenergic receptor agonists on electrical field-stimulated contractions of isolated bronchi in horses. *J Vet Pharmacol Ther.* 2017.
- [13] Menozzi A, Pozzoli C, Poli E, Delvescovo B, Serventi P, Bertini S. Pharmacological characterization of muscarinic receptors in the contractions of isolated bronchi in the horse. *J Vet Pharmacol Ther.* 2014;37:325-31.
- [14] Cazzola M, Calzetta L, Puxeddu E, Ora J, Facciolo F, Rogliani P, et al. Pharmacological characterisation of the interaction between glycopyrronium bromide and indacaterol fumarate in human isolated bronchi, small airways and bronchial epithelial cells. *Respir Res.* 2016;17:70.
- [15] Calzetta L, Soggiu A, Roncada P, Bonizzi L, Pistocchini E, Urbani A, et al. Propofol protects against opioid-induced hyperresponsiveness of airway smooth muscle in a horse model of target-controlled infusion anaesthesia. *Eur J Pharmacol.* 2015;765:463-71.

- [16] Calzetta L, Rogliani P, Mattei M, Alfonsi P, Cito G, Pistocchini E, et al. Pharmacological characterization of the interaction between tiotropium and olodaterol administered at 5:5 concentration-ratio in equine bronchi. *COPD*. 2017;14:526-32.
- [17] Matera MG, Calzetta L, Rogliani P, Bardaro F, Page CP, Cazzola M. Evaluation of the effects of the R- and S-enantiomers of salbutamol on equine isolated bronchi. *Pulm Pharmacol Ther*. 2011;24:221-6.
- [18] Matera MG, Calzetta L, Peli A, Scagliarini A, Matera C, Cazzola M. Immune sensitization of equine bronchus: glutathione, IL-1 β expression and tissue responsiveness. *Respir Res*. 2005;6:104.
- [19] Calzetta L, Cazzola M, Page CP, Rogliani P, Facciolo F, Matera MG. Pharmacological characterization of the interaction between the dual phosphodiesterase (PDE) 3/4 inhibitor RPL554 and glycopyrronium on human isolated bronchi and small airways. *Pulm Pharmacol Ther*. 2015;32:15-23.
- [20] Cazzola M, Calzetta L, Facciolo F, Rogliani P, Matera MG. Pharmacological investigation on the anti-oxidant and anti-inflammatory activity of N-acetylcysteine in an ex vivo model of COPD exacerbation. *Respir Res*. 2017;18:26.
- [21] Manzini S. Bronchodilatation by tachykinins and capsaicin in the mouse main bronchus. *Br J Pharmacol*. 1992;105:968-72.
- [22] Meini S, Ballati L, Evangelista S, Manzini S. Isbufylline, a xanthine derivative, inhibits bronchoconstrictor responses produced by stimulation of capsaicin-sensitive sensory nerves in guinea-pig: 'In vitro' and 'in vivo' evidence. *Pulm Pharmacol*. 1993;6:279-86.
- [23] LeBlanc PH, Eberhart SW, Robinson NE. In vitro effects of alpha 2-adrenergic receptor stimulation on cholinergic contractions of equine distal airways. *Am J Vet Res*. 1993;54:788-92.
- [24] Matera MG, Calzetta L, Sanduzzi A, Page CP, Cazzola M. Effects of neuraminidase on equine isolated bronchi. *Pulm Pharmacol Ther*. 2008;21:624-9.
- [25] Olszewski MA, Robinson NE, Zhu FX, Zhang XY, Tithof PK. Mediators of anaphylaxis but not activated neutrophils augment cholinergic responses of equine small airways. *Am J Physiol*. 1999;276:L522-9.
- [26] Gunthorpe MJ, Rami HK, Jerman JC, Smart D, Gill CH, Soffin EM, et al. Identification and characterisation of SB-366791, a potent and selective vanilloid receptor (VR1/TRPV1) antagonist. *Neuropharmacology*. 2004;46:133-49.
- [27] Rizzo CA, Valentine AF, Egan RW, Kreutner W, Hey JA. NK(2)-receptor mediated contraction in monkey, guinea-pig and human airway smooth muscle. *Neuropeptides*. 1999;33:27-34.
- [28] Calzetta L, Spina D, Cazzola M, Page CP, Facciolo F, Rendina EA, et al. Pharmacological characterization of adenosine receptors on isolated human bronchi. *Am J Respir Cell Mol Biol*. 2011;45:1222-31.
- [29] Motulsky H, Christopoulos A. Fitting models to biological data using linear and nonlinear regression : a practical guide to curve fitting. Oxford: Oxford University Press; 2004.
- [30] Schild HO. pA, a new scale for the measurement of drug antagonism. *Br J Pharmacol*. 1997;120:29-46; discussion 27-8.
- [31] Owen SC, Doak AK, Ganesh AN, Nedyalkova L, McLaughlin CK, Shoichet BK, et al. Colloidal drug formulations can explain "bell-shaped" concentration-response curves. *ACS Chem Biol*. 2014;9:777-84.

- [32] Tucek S, Michal P, Vlachova V. Modelling the consequences of receptor-G-protein promiscuity. *Trends Pharmacol Sci.* 2002;23:171-6.
- [33] Rogliani P, Calzetta L, Rendina EA, Massullo D, Dauri M, Rinaldi B, et al. The influence of propofol, remifentanyl and lidocaine on the tone of human bronchial smooth muscle. *Pulm Pharmacol Ther.* 2013;26:325-31.
- [34] Koyama S, Rennard SI, Claassen L, Robbins RA. Dibutyryl cAMP, prostaglandin E2, and antioxidants protect cultured bovine bronchial epithelial cells from endotoxin. *Am J Physiol.* 1991;261:L126-32.
- [35] Moreland JG, Fuhrman RM, Wohlford-Lenane CL, Quinn TJ, Benda E, Pruessner JA, et al. TNF-alpha and IL-1 beta are not essential to the inflammatory response in LPS-induced airway disease. *Am J Physiol Lung Cell Mol Physiol.* 2001;280:L173-80.
- [36] Tamaoki J, Tagaya E, Yamawaki I, Sakai N, Nagai A, Konno K. Effect of erythromycin on endotoxin-induced microvascular leakage in the rat trachea and lungs. *Am J Respir Crit Care Med.* 1995;151:1582-8.
- [37] De Winter BY, Bredenoord AJ, Van Nassauw L, De Man JG, De Schepper HU, Timmermans JP, et al. Involvement of afferent neurons in the pathogenesis of endotoxin-induced ileus in mice: role of CGRP and TRPV1 receptors. *Eur J Pharmacol.* 2009;615:177-84.
- [38] Bret-Dibat JL, Kent S, Couraud JY, Creminon C, Dantzer R. A behaviorally active dose of lipopolysaccharide increases sensory neuropeptides levels in mouse spinal cord. *Neurosci Lett.* 1994;173:205-9.
- [39] Takemura M, Quarcoo D, Niimi A, Dinh QT, Geppetti P, Fischer A, et al. Is TRPV1 a useful target in respiratory diseases? *Pulm Pharmacol Ther.* 2008;21:833-9.
- [40] Reilly CA, Johansen ME, Lanza DL, Lee J, Lim JO, Yost GS. Calcium-dependent and independent mechanisms of capsaicin receptor (TRPV1)-mediated cytokine production and cell death in human bronchial epithelial cells. *J Biochem Mol Toxicol.* 2005;19:266-75.
- [41] Belvisi MG, Barnes PJ. TRPV1 in the airways. Turning up the Heat on Pain: TRPV1 Receptors in Pain and Inflammation. 2005:167-87.
- [42] Nasra J, Belvisi MG. Modulation of sensory nerve function and the cough reflex: understanding disease pathogenesis. *Pharmacol Ther.* 2009;124:354-75.
- [43] Papoiu AD, Yosipovitch G. Topical capsaicin. The fire of a 'hot' medicine is reignited. *Expert Opin Pharmacother.* 2010;11:1359-71.
- [44] Widdicombe JG. Autonomic regulation. i-NANC/e-NANC. *Am J Respir Crit Care Med.* 1998;158:S171-5.
- [45] Matera MG, Amorena M, Lucisano A. Innervation of equine airways. *Pulm Pharmacol Ther.* 2002;15:503-11.
- [46] Venugopal CS, Holmes EP, Polikepahad S, Laborde S, Kearney M, Moore RM. Neurokinin receptors in recurrent airway obstruction: a comparative study of affected and unaffected horses. *Can J Vet Res.* 2009;73:25-33.
- [47] Belvisi MG. Airway sensory innervation as a target for novel therapies: an outdated concept? *Curr Opin Pharmacol.* 2003;3:239-43.
- [48] Belvisi MG. Sensory nerves and airway inflammation: role of A delta and C-fibres. *Pulm Pharmacol Ther.* 2003;16:1-7.
- [49] Joos GF, Van Schoor J, Kips JC, Pauwels RA. The effect of inhaled FK224, a tachykinin NK-1 and NK-2 receptor antagonist, on neurokinin A-induced bronchoconstriction in asthmatics. *Am J Respir Crit Care Med.* 1996;153:1781-4.

- [50] Kraan J, Vink-Klooster H, Postma DS. The NK-2 receptor antagonist SR 48968C does not improve adenosine hyperresponsiveness and airway obstruction in allergic asthma. *Clin Exp Allergy*. 2001;31:274-8.
- [51] Van Schoor J, Joos GF, Chasson BL, Brouard RJ, Pauwels RA. The effect of the NK2 tachykinin receptor antagonist SR 48968 (saredutant) on neurokinin A-induced bronchoconstriction in asthmatics. *Eur Respir J*. 1998;12:17-23.
- [52] Ichinose M, Nakajima N, Takahashi T, Yamauchi H, Inoue H, Takishima T. Protection against bradykinin-induced bronchoconstriction in asthmatic patients by neurokinin receptor antagonist. *Lancet*. 1992;340:1248-51.
- [53] Boot JD, de Haas S, Tarasevych S, Roy C, Wang L, Amin D, et al. Effect of an NK1/NK2 receptor antagonist on airway responses and inflammation to allergen in asthma. *Am J Respir Crit Care Med*. 2007;175:450-7.
- [54] Schelfhout V, Louis R, Lenz W, Heyrman R, Pauwels R, Joos G. The triple neurokinin-receptor antagonist CS-003 inhibits neurokinin A-induced bronchoconstriction in patients with asthma. *Pulm Pharmacol Ther*. 2006;19:413-8.
- [55] Joos GF, Vincken W, Louis R, Schelfhout VJ, Wang JH, Shaw MJ, et al. Dual tachykinin NK1/NK2 antagonist DNK333 inhibits neurokinin A-induced bronchoconstriction in asthma patients. *Eur Respir J*. 2004;23:76-81.